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L. M. Mosulishvili*, M. V. Frontasyeva,
A. I. Belokobylsky*, E. I. Kirkesali*, A. I. Khizanishvili*,
E. V. Pomyakushina

# EPITHERMAL NEUTRON ACTIVATION ANALYSIS OF *SPIRULINA PLATENSIS* BIOMASS, OF THE C-PHYCOCIANIN AND OF DNA EXTRACTED FROM IT

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*E. L. Andronikashvili Institute of Physics, Georgian Academy of Sciences, Tbilisi, Georgia

## Introduction

The current environmental condition deteriorations, mental and physical stress, charges in the diet have become serious risk factors for the human organism, increased the death rate and civilizations diseases.

These are the obvious reasons why new progressive trends are being extensively developed in modern medicine, pharmacology, and biotechnology and more effective harmless medicaments are being sought for to treat and prevent various diseases.

One of trends in biotechnology is associated with the blue-green microalga *Spirulina platensis*, which has been widely used since the 1990s. The biomass of Spirulina and its processing products are employed as feed and food additives in agriculture, food industry, pharmaceutics, perfumery making, medicine, and science.

It is characterized by high protein content (60-70%), non-toxity, well amino acid composition, richness in vitamins and different biologically active agents [1].

Spirulina is held to work profitably as an immunopotentiator and is characterized by the anticarcinogenic and antiviral effects.

Spirulina is often used before and in the course of drug treatment to remove harmful agents from the organism and to introduce a variety of vital biologically active elements and compounds, which results in normalizing of metabolism and strengthening of the immune system, *i.e.* favourable conditions for the action of drugs are provided. An example is the use of Spirulina to treat children who suffered from the Chernobyl accident.

The investigations showed that during cultivation of spirulina the some trace elements are built into organic molecules. Then the biomass of cells may be used as a matrix for production the pharmaceuticals to treat illnesses stemming from lack of some elements and their compounds in the organism [2].

The ability to biotransform and endogenously add the desired elements (Se, I, Cr, *etc.*) producing complexes easily assimilated by a human organism is a distinctive feature of *Spirulina platensis*. Being a living organism, spirulina accumulates elements strictly as much as is necessary for the organism. Spirulina-based preparations contain a complex of biologically active agents and produce both therapeutic and health-improving effect, to develop new pharmaceuticals the precise analytical control at all technological stages is of great importance.

## Experimental

### *Analysis of Spirulina biomass*

Neutron activation analysis was chosen for this purpose as the most suitable method for these investigations. The method of the instrumental neutron activation analysis in its epithermal-neutron version (ENAA) for studying the multi–element composition of *Spirulina platensis* was used earlier [3].

The neutron activation analysis for to study *Spirulina platensis* biomass was also described in works of the Italian scientists [4,5]. Therefore, the composition of *Spirulina platensis* was studied by ENAA widely used at the pulsed fast reactor IBR-2 (JINR FLNP, Dubna) with a very high epithermal-to-thermal neutron ratio. The characteristics of irradiation channels connected with the pneumatic transport system of the IBR-2 reactor at JINR FLNP are present in [6].

The *Spirulina platensis* IPPAS B-265 strain from Timiriazev Institute for Plant Physiology of the Russian Academy of Sciences was cultivated for experiments. To study background concentrations of various elements in the *Spirulina platensis* biomass, cultivation was carried out in a standard nutrient medium with distilled and drinking water.

Spirulina grows well in a standard alkaline mineral nutrient medium at a temperature of 30-34 °C, pH 8.5-11, under sodium lamp light. The maximum growth of cells occurs on the 4-5 th day of cultivation.

After cultivation the Spirulina cell mass was separated from the nutrient, washed with distilled water three times and centrifuged. The resulting wet biomass was lyophilically dried in a adsorption-condensation lyophilizer. The native dry biomass for analysis was made into small pellets of various diameters and thickness by means of a special titanium mould. Samples weighing some 50 mg were packed in aluminum foil to determine long-lived isotopes and in polyethylene to determine short-lived isotopes.

To determine long-lived isotopes, irradiation channel Ch1 was used. The samples were irradiated for five days, repacked and measured twice, after being kept for 4 and 20 days. The measurement time varied from 1.5 to 10 hours. To determine short-lived Mg, Al, Cl, Ca, V, Mn and I isotopes, irradiation channel Ch2 was used. Samples were irradiated for three minutes and measured two times, after being kept for 3-5 and 20 minutes. The measurement time was 5-8 and 20 min respectively. Gamma-ray spectra were registered using a large volume Ge(Li) detector with a resolution of 1.9 keV at the 1332.4 keV line of $^{60}$Co, with an efficiency of 30% relative to a 3x3" NaI detector for the same line. The data were processed and the element concentrations were determined with certified reference items normally used at the laboratory [3].

Three certified standards, IAEA Lichen-336, IAEA Bottom Sediments SDM-2T and Danish Moss DK-1, were used to control the quality of analytical measurements. In addition, synthetic many-element standards SSB-1 and SSB-2 for NAA of biological samples [7], locally developed on the basis of phenol-formaldehyde resin, were used.

The elemental composition of biomass of *Spirulina platensis* was determined by method of ENAA.

A total of 27 macro-, micro- and trace elements were determined for the experimental samples (cultivated in a standard nutrient medium with distilled and drinking water). The results are shown in Table 1, where the elements are given in order of descending concentrations within seven orders of magnitude ($10^{-3}$ – $10^{+4}$ ppm) from macroelements to ultra trace elements.

As is evident from the table, concentrations of toxic elements (Hg, As, Cr, *etc*) in the *Spirulina platensis* biomass are in the order of μg/g. Trace amounts of these

elements apper in chemical reagents used to prepare a nutrient. Therefore, highly pure chemicals must be used to produce biomass for pharmaceutical purposes.

On the other hand, the US data on permissible doses of various elements for a human organism (see http://www.spirulina.com /SPBNutrition.html) show that our results obtained with the reagents of the chemically pure and grade do not exceed the permissible level.

*Table 1.* Concentration of elements in the *Spirulina platensis* biomass

| Element | Concentration (dist. water), ppm | Error, % | Concentration (drink. water), ppm | Error, % |
|---|---|---|---|---|
| K | 18025 | 10 | 26620 | 10 |
| Na | 15485 | 15 | 28090 | 15 |
| Cl | 5686 | 10 | 8200 | 10 |
| Mg | 1640 | 20 | 4666 | 20 |
| Fe | 1356 | 10 | 1224 | 10 |
| Ca | 937 | 25 | 759 | 25 |
| Zn | 115 | 16 | 44 | 20 |
| Al | 94 | 12 | 102 | 14 |
| Mn | 48 | 10 | 41 | 10 |
| W | 11 | 10 | 0.4 | 20 |
| Cr | 6.2 | 14 | 3.8 | 18 |
| Ni | 4.7 | 27 | 2.7 | 28 |
| Ba | 4.2 | 10 | 10.4 | 10 |
| Co | 0.98 | 10 | 0.75 | 10 |
| Br | 0.7 | 20 | 0.3 | 20 |
| As | 0.57 | 30 | 0.61 | 30 |
| V | 0.4 | 20 | 0.3 | 20 |
| Ag | 0.39 | 15 | 0.27 | 15 |
| Rb | 0.37 | 16 | 0.31 | 14 |
| I | 0.36 | 30 | 6.8 | 15 |
| Mo | 0.14 | 20 | 0.22 | 20 |
| Tm | 0.111 | 13 | 0.042 | 15 |
| Sb | 0.10 | 12 | 0.08 | 11 |
| Au | 0.016 | 20 | 0.028 | 30 |
| Sc | 0.01 | 20 | 1.38 | 20 |
| Ta | 0.006 | 20 | 0.002 | 20 |
| Hg | 0.004 | 30 | 0.003 | 30 |

*Analysis of C-phycocianin*

The ENAA method was applied to study the elemental content of C-phycocyanin (C-PC) extracted from Spirulina with different grade of purity. C-PC – a special pigment of phicobilin kind which takes an active part in photosynthesis. It absorbs light with a wavelength of 620 nm with a maximum efficiency of order 70% and it emits waves with a length of 647 nm.

In Spirulina biomass consisting to 55-70% of proteins playing a vitally important role, the portion of C-PC is 14% on average.

C-PC stimulates the immune system is thus a promising means in the treatment of the different kinds of cancer or AIDS.

In connection with a great scientific and applied interest in C-phycocianin-based products there are being carried out intense investigations of the different levels of its physicochemical properties of importance for the use of C-phycocianin for the purposes of medical treatment and prophylaxis of diseases [8].

C-PC was extracted from the *Spirulina platensis* biomass using a modified version of the Teal and Dale method [9]. The procedure involves a cycle of protein purification operations accompanied with spectrophotometric purity checks of the preparation by the ratio of absorption peaks at $\lambda$=620 and 280 nm ($D_{620}/D_{280}$). If $D_{620}/D_{280}>4$, the C-PC preparation is considered to have a high putity.

The obtained C-PC preparation was freeze-dried and then formed into 50 mg pellets to conduct neutron activation analysis.

The ENAA method was applied to study pellet samples of native *Spirulina platensis* biomass, low purity C-PC ($D_{620}/D_{280}$=2.04) and of high purity C-PC ($D_{620}/D_{280}\geq4$).

The results obtained for long-lived isotopes are summarized in Table 2. The sample irradiation time was 5 days. After irradiation the samples were rewrapped and measured twice after 4 and 20 days. The measuring time for the different elements varied from 1.5 to 10 hours.

*Table 2.* The ENAA results of *Spirulina platensis* and C-PC extract samples [10]

| Element | Content in *Spirulina pl.* Biomass, mg/kg | Error, ±% | Content in low purity C-PC, mg/kg | Error, ±% | Content in high purity C-PC, mg/kg | Error ±% |
|---|---|---|---|---|---|---|
| K | 20000 | 8 | 183 | 20 | 287800 | 10 |
| Na | 13050 | 15 | 1017 | 10 | 42770 | 15 |
| Fe | 3800 | 20 | 1400 | 8 | 2200 | 10 |
| Sr | 1< | - | 3.950 | 33 | 77 | 30 |
| Cr | 5.6 | 10 | 41.00 | 16 | 48 | 15 |
| Mo | 0.39 | 20 | 5.510 | 5 | 11 | 4 |
| As | 0.57 | 30 | 0.19 | 11 | 10 | 11 |
| Ni | 1.3 | 11 | 14.300 | 14 | 9 | 20 |
| Au | 0.068 | 15 | 0.00814 | 5 | 7 | 20 |
| Ag | 0.63 | 15 | 0.72 | 10 | 6.7 | 20 |
| Ba | 9.80 | 10 | 42 | 10 | 5.9 | 15 |
| Rb | 0.12 | 12 | 0.146 | 20 | 4.0 | 12 |
| Co | 0.10 | 13 | 0.018 | 10 | 3.1 | 10 |
| Br | 0.53 | 12 | 0.530 | 20 | 0.7 | 5 |
| Hg | 0.0035 | 30 | - | - | 0.61 | 30 |
| Sb | 0.065 | 10 | 0.219 | 10 | 0.55 | 16 |
| Se | 0.1< | | - | - | 0.08 | 27 |
| Zn | 10 | 12 | 68.3 | 10 | 385 | 14 |
| W | 2.5 | 10 | 0.408 | 8 | 0.5 | 50 |
| Sm | 0.0054 | 10 | 0.0023 | 25 | 0.1 | 40 |

Increased concentrations of K and Na in the samples of pure C-PC are due to that the C-PC solution was freeze-dried in a Na-K phosphate buffer at pH 6.0.

The assessment of elements concentrations in C-phycocianin after its purification makes it possible to suggest which of the studied metals could take part in the formation of macromolecular complexes with C-phycocianin of the protein-metal-chromophore type.

It should be noted that a comparative estimation of the concentrations of metals in the investigated preparations was performed accounting for the fact that C-PC is a constituent part of proteins composing 55-70% of the spirulina biomass and its portion is 14% of the total amount of the proteins.

According to estimation taking into account the percentage of C-PC metals in *Spirulina platensis* may follow the sequence: Zn>Cr>Ni>Co>As>Sr>Mo>Ag>Hg.

The content of such toxic metals as Hg, As, Sr, etc. does not exceed the presently accepted off-limit for the human organism (see http://www.spirulina.com).

Thus, the obtained C-PC preparations can be used for the purposes of pharmacology both in a pure form and after being purposely loaded with some elements.

*Analysis of DNA*

The method of ENAA also was used for study the content of metals in the desoxyribonucleic acid (DNA), extracted from *Spirulina platensis* (Table 3).

*Table 3.* Concentration of metals in DNA extracted from *Spirulina platensis*

| Element | Concentration, ppm | Error, % |
|---|---|---|
| Na | 30300 | 10 |
| K | 4850 | 15 |
| Ca | 1260 | 40 |
| Fe | 321 | 20 |
| Al | 213 | 10 |
| Zn | 21 | 10 |
| Br | 3.4 | 15 |
| Ni | 1.4 | 20 |
| Ba | 0.18 | 15 |
| Sb | 0.18 | 20 |
| Ag | 0.10 | 40 |
| Cs | 0.09 | 25 |
| Co | 0.08 | 15 |
| Au | 0.003 | 40 |

DNA preparations were isolated from fresh Spirulina pl. biomass by combined method of A.A.Linskay [11] and A.Vonshak [1], recommendations of E.C.Travaglini [12] and G.P.Miroshnichenko [13] included. Newly harvested Sp.pl. biomass was washed in distilled water, suspended in 0.15 M NaCl-0.1M EDTA, pH 8.0, then frozen in liquid nitrogen and melted at 37°C. The procedure of freezing-melting was repeated thrice. Then there followed stages of final lysis of Spirulina pl. cells in lysozyme, deproteinization by sodium dodecylsulfate and proteinase K, protein removal by mixture of phenol-chloroform-isoamyl ethanol. The release from RNA was performed by treatment of unpurified DNA solution with RNAase, by additional separation of proteins with mixture of phenol-chloroform-isoamyl ethanol.

DNA was precipitated in isopropanol, then in ethanol, the pellet was washed by increasing concentrations of ethanol and was dried under vacuum.

DNA isolated by this technique has good spectral characteristics $A_{230}/A_{260} \approx 2$ and its melting temperature equals 86.9 °C. The irradiation and measurement for long lived and short lived isotopes was carried out in analogous of described above.

According to our results, it can be assumed what metals is bound in the structural systems of DNA and these results can be used for future investigations.

## Conclusions

1. Composition of the blue-green microalga *Spirulina platensis* biomass is studied. Concentrations of 27 macro- and microelements are found in a wide range by the ENAA method. It is shown that the Spirulina biomass cultivated as proposed does not incorporate toxic elements in concentrations higher than permissible and can be used as a matrix for production of pharmaceuticals.
2. It is established that some of the investigated metals (Zn, Ni, Sr, Cr, Co, Mo, etc.) are still present in C-PC after it is purified, which means that they could be present in the composition of macromolecular complexes with C-PC of the protein- metal-chromophore type.
3. It is shown that the content of toxic metals in C-PC does not exceed the presently accepted off-limit and C-PC can be thus used, both in a pure form and in complex with appropriate elements, to prepare pharmaceuticals.
4. Concentration of 14 metals by ENAA method was determined in DNA extracted from *Spirulina platensis.*

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Эпитепловой нейтронный активационный анализ биомассы *Spirulina platensis* и выделенных из нее С-фикоцианина и ДНК

Метод эпитеплового нейтронного активационного анализа (ЭНАА) был использован для изучения состава биомассы *Spirulina platensis*. Определен фоновый уровень концентраций 27 макро-, микро- и следовых элементов в пределах от $10^{-3}$ до $10^{4}$ мкг/г. Установлено, что биомасса спирулины не содержит токсичных элементов выше пределов допустимых уровней и может быть использована для изготовления фармацевтических препаратов на ее основе. Методом ЭНАА определены концентрации основных элементов в С-фикоцианине и ДНК, выделенных из *Spirulina platensis*. Проведено сравнение элементного состава биомассы спирулины с составом очищенного С-фикоцианина.

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Epithermal Neutron Activation Analysis of *Spirulina platensis* Biomass, of the C-Phycocianin and of **DNA** Extracted from It

The epithermal neutron activation analysis (ENAA) was used for study of the biomass of *Spirulina platensis*. The background levels of concentration 27 macro-, micro- and trace elements ranging from $10^{-3}$ up to $10^{4}$ ppm were determined. It was found that the biomass of spirulina does not contain toxic elements above the tolerance levels and can be utilized as a matrix of pharmaceuticals based on it.

The concentrations of basic elements in C-phycocianin and DNA extracted from *Spirulina platensis* were determined by ENAA. A comparison of the element content of a whole spirulina biomass with that of a refined C-phycocianin preparation was made.

The investigation has been performed at the Frank Laboratory of Neutron Physics, JINR and at the E. L. Andronikashvili Institute of Physics of the Georgian Academy of Sciences.

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